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Molecular detection, isolation and characterization of avian rotavirus from broiler chicken

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SUMMARY

Chicken rotaviruses (CRVs) were detected, identified and characterized in broiler chicken with diarrhea for the first time in Egypt. Fecal samples were collected from 85 naturally occurring diarrheal outbreaks in commercial chicken broilers farms that are located in a wide range of geographical areas including many Egyptian governorates mainly 6th Octobar, El-fayom, Giza, Qaluobea, Menofia and El-mansoura during year 2008. CRV was detected in the fecal samples by ELISA using monoclonal antibodies (Mabs) against VP6, Electron Microscopy (EM), Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and the virus was isolated using SPF chickens. The obtained results confirmed the isolation and identification of group A chicken rotavirus while the molecular characterization analysis using different primers sets suggested that the isolated chicken rotavirus does not belong to the same cluster of Ch1 rotavirus strain but most likely more related to Po-13 strain (mammalian like chicken rotavirus strains). This study reports the importance of rotaviruses in broiler chickens with delayed growth and diarrhea.

INTRODUCTION

Rotaviruses are the causes of enteric disease in a wide variety of avian and mammalian species, including human beings (Tzipori, 1985). They were first identified in avian species in 1977 by Bergeland et al., (1977) who reported them as causes of enteritis in turkey poults. Since that time, they have been identified in both domestic and free-ranging avian species including chickens, pheasants, and ducks (Gough et al., 1985 and 1986; McNulty, 2003; Reynolds et al., 1987a and b).

Rotaviruses are classified as a separate genus within family Reoviridae (Estes, 1990). Rotaviruses are nonenveloped, spherical and

have a diameter of approximately 70 nm. Intact viruses consist of two icosahedral capsid shells (approximately 50 and 70 nm in diameter); they have a distinctive "wheel-like" appearance by negative-stain EM owing to a smooth outer rim and capsomeres of the inner capsid that radiate toward the rim. The genome is comprised of 11 linear segments of double-stranded (ds) RNA. The rotavirus virion consists of a three concentric protein shells, or layers. The inner most layer, or core, is composed of the VP2 protein, the middle layer is composed of the VP6 protein, and the outer layer is composed of two neutralizing proteins; the major surface glycoprotein, VP7 (glycoprotein, G) and haemagglutinin spike, VP4 (Protease sensitive, P); (Estes and Cohen, 1989).

Classification of avian rotaviruses has been based primarily on cross-immunofluorescence studies and PAGE analyses of dsRNA segments. Avian rotaviruses that cross-react by fluorescent antibodies (FA) with antisera prepared against Group A mammalian rotaviruses are classified as Group A avian rotaviruses (McNulty et al., 1979a). Rotaviruses that lack the Group A antigen are referred to as "atypical" rotaviruses, including those avian rotaviruses designated as groups D, F, and G (McNulty et al., 1981 and McNulty, 2003). Yet, avian rotaviruses from group A have already been isolated from the intestinal contents of chickens, turkeys, and other avian species (Brüssow et al., 1992b, Sugiyama et al., 2004).

In field conditions, rotavirus infections in poultry may induce subclinical manifestations, or they may be associated with enteritis, dehydration, anorexia, low weight gain, and increased mortality (McNulty, 2003; Tamehiro et al., 2003). In both layer hens and broiler chickens, rotavirus has already been established as the etiological agent of enteritis, originated from viral replication in intestinal epithelium, resulting in diarrhea and nutrient malabsorption (Snodgrass et al., 1986), which causes an increase in feed conversion ratio and large economic losses to poultry industry (Barnes, 1997).

Diagnosis of rotavirus infection usually is based on detection of viruses in feces using EM, detection of viral antigens in tissues using FA, or demonstration of rotavirus RNA in feces using PAGE. Detection of rotavirus in feces by direct EM is a sensitive diagnostic approach and this method will detect rotaviruses of all serogroups (Theil et al., 1986). Immune EM and FA require specific antisera; however, these procedures may be used to identify specific serogroups. RT-PCR and ELISA had been also used for diagnosis and characterization of rotavirus in fecal samples (Hussein et al., 1995 and 1996).

The lack of information on the presence of chicken rotaviruses in Egyptian

broilers was the major motivation for this work. The aim of the present study was focused on detection, isolation and identification of chicken rotaviruses.

MATERIAL AND METHODS

Fecal samples:

85 fecal samples were collected from diarrheic chickens from 1 to 6 weeks of age with a special focus on age between 20 and 32 days in order to diminish the effect of the maternal immunity on virus shedding and also to avoid the ages of solid immunity development that linked with higher ages with different degrees of severity. Samples were collected from different poultry farms representing a wide range of geographical areas distribution including many Egyptian governorates mainly 6th October, El-Fayom, Giza, Oaluobea, Menofia and El-Mansoura.

VP6 Mabs-based ELISA:

Fecal samples were tested for group A rotavirus by Mabs–based ELISA according to Hussein et al., (1995). Briefly, ELISA plates were coated with 3.4 ug/well of Rotavirus specific polyclonal antibodies. The plate was then incubated for 1 hour at 37°C. Non-specific binding sites were saturated with 10% blocking buffer (non fat dry milk (NFDM) in PBS pH 7.4) over night at 4°C. After washing with PBS containing 0.05% Tween-20 (PBS-T), the chicken fecal samples diluted PBS were added to plate and incubated at 37°C for 1 hour then washed again 3 times. VP6 Mabs was added then the plate was incubated at 37°C for 1 hour then washed again 3 times. Secondary antibody (antimouse conjugated peroxidase) were added to each well then incubated at 37°C for 1hour before thoroughly 3 times washing with PBS-T. The color was developed with the addition of H_2O_2/OPD for 20 min. Further color development was stopped by SDS (0.5%), and then the plate was read at 450 nm wavelength.

Electron microscopy:

Positive fecal samples in ELISA were examined with Negative-Stain Electron Microscopy (McNulty et al. 1979b) to detect the characteristic feature of rotavirus particles. Examination of the satined samples was carried out at the EM unit VACSERA, Agouza, Giza, Egypt.

Isolation of CRV in SPF chicks

Groups of SPF chickens aged 14, 28 and 38 days were infected orally with 1 ml of the prepared fecal sample suspension. Age-matched SPF chickens were kept as controls. At daily intervals after 72 hours, fecal samples were collected from the experimentally infected chicks; representative birds from each group were sacrificed and examined.

Extraction of viral nucleic acids:

The CRV double-stranded RNA (dsRNA) was extracted from fecal samples using Trizol RNA extraction kit [GIBCO] according to recommended procedures (Chomeznski and Sacchi, 1987). The reagent is

a mono-phasic solution of phenol and guanidine isothiocyanate for RNA isolation in a single step that *maintains* the integrity of the RNA, while disrupting cells and dissolving cell components. The aqueous chloroform supernatant containing RNA was collected then washed and precipitated with isopropanol followed by ethanol 75%. The extracted RNA was suspended in nuclease free water and kept at -85 ◦C till used for RT-PCR.

RT-PCR amplification:

The CRV dsRNA was RT-PCR amplified as described previously (El-sabagh et al., 2007). The primers for RT-PCR were as follows: Mammalian VP6 forward primer 5′-GGCTTTTAAACGAAGTCTTCAACATGG-3′ (nucleotide 1- 27) and Mammalian VP6 reverse primer 5′-GGTCACATCCTCTCACTACGC-3′ (complementary to nucleotide 1336- 1356), Mammalian VP7 forward primer, 5′-GCGGTTAGCTCCTTTTAATGTATGG-3′ (nucleotide 32- 56) and Mammalian VP7 reverse primer 5′-GGTCACATCATACAACTCTAATCTAACA TG-3′ (complementary to nucleotide 1033-1062) (El-sabagh, 2006). Chicken (Ch1) VP6 forward primer 5′-GGCTTTTAAACGAAGTCTTC-3′ (nucleotide 1-20) Chicken (Ch1) VP6 reverse primer 5′-GGTCACATCCTCTCACTA-3′ (complementary to nucleotide 1331-1348) (Elschner et al., 2005). Pigeon (Po-13) VP6 forward primer 5′-GGAATTTGCAAAATGGAAGA-3′ (nucleotide 440-459) and Pigeon (Po-13) VP6 reverse primer 5′-GCTGGTGTCATATTTGGT-3′ (complementary to nucleotide 915-932) (Ito et al., 2001).The RT-PCR products were analyzed on 1.25% agarose gel containing 0.5 ug/ml ethidium bromide.

RESULTS

Antigenic Detection of CRVs using VP6 Mabs based-ELISA:

ELISA, Based on the use Mabs reacted with the VP6 of all groups A RV, was carried out to identify the rotavirus in the direct fecal sample suspension. The screening results of the captured double sandwich ELISA showed 13 positive samples out of 85 total tested samples with prevalence of 15.2 %. Table (1) summarize the screening results of the captured sandwich VP6 Mabs based-ELISA for the examined fecal samples and summarize the age and governmental locations of positive fecal samples.

Molecular detection of CRVs using VP6 and VP7 specific mammalian primers:

7 samples showed the highest optical densities out of 13 positive samples in VP6 Mabs based-ELISA were selected to be used in RT-PCR detection of rotaviruses RNA using VP6 and VP7 mammalian primers. The results of RT-PCR revealed that only one sample showed the

1356bp specific band of VP6 gene while the VP7 primers did not show any specific bands.

Detection of CRVs using Negative Stain Electron Microscopy:

The fecal sample showed VP6 specific band in RT-PCR was clarified by low speed centrifugation and concentrated through ultra-centrifugation then examined using negative stain electron microscopy. Fig. (1) Show both double-layered and triple-layered rotavirus particles in the original field sample. This field sample was orally administered to SPF chicks then the fecal samples were collected from the experimentally infected chicks and examined again using EM. Fig. (2) Show the rotavirus particles in fecal samples collected from SPF chicks

Table (1): The positivity degree of the positive fecal chicken samples as compared with positive and negative controls.

Key No. of tested samples	Age	Governmental location	Positive degree
12	28 days	Qaluobea	+
13	28 days		+
16	22 days		++
18	12 days		++
20	20 days		+
52	20-30 days	Mansoura	+
60			+
67			++
68			+
69			+
78			+
81			++
85			+
NCDV strain	Standard MA-104 tissue culture adapted positive control rotavirus		+++
UK strain			+++
PBS	Negative control diluents		-
MEM			-

Molecular detection of CRVs in feces of experimentally infected SPF chicks using VP6 specific chicken primers:

Two sets of CRVs VP6 gene specific primers were designed. The first set amplify 1348 bp fragment (Ch1strain) while the second set amplify 470 bp fragment (Po-13 mammalian like strains). Analysis of the RT-PCR products in ethidium bromide-stained agarose gel revealed that the tested fecal

samples collected at day 4 and day 5 post experimental infection in SPF chicks were negative with Ch1 specific primer and positive with Po-13 mammalian like strains.

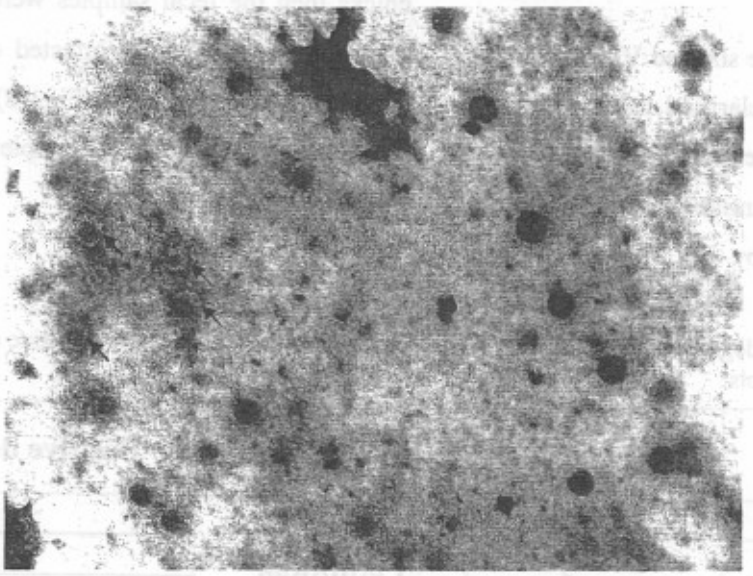

Fig. (1): EM of rotavirus detected in field chicken fecal samples. Note the wheel-like appearance of some of the rotavirus particles. Magnification is 30.000 times.

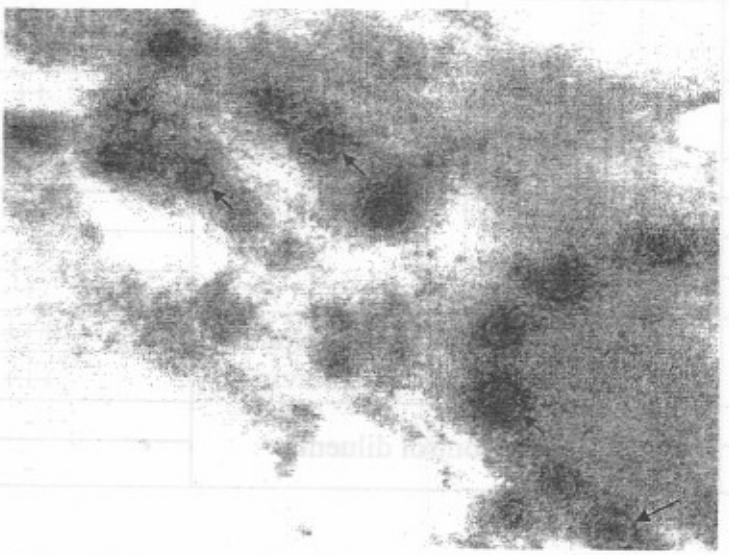

Fig. (2): EM of chicken rotaviruses after isolation in SPF chicks. Note the presence of shelled particles detected in 5th day collected fecal samples from experimentally. Magnification is 40.000 times.

DISCUSSION

Rotavirus replication occurs primarily in mature villus epithelium of the small intestine (McNulty, 1997), and maximum excretion of virus in feces occurs from 2 to 5 days postexposure (McNulty et al., 1983). Rotaviruses gain entry to the body through ingestion, and transmission occurs via fecal shedding (McNulty et al., 1983) so the 85 fecal samples were collected from diarrheic chickens suspecting rotavirus infection. The collected samples were representing most of common commercial Egyptian broiler and from multiple ages ranging from 1 to 6 weeks with a special focus on age between 20 and 32 days in order to diminish the effect of the maternal immunity and also to avoid the ages of solid immunity development that linked with higher ages.

ELISA was more efficient and sensitive than other detection tests (Ellen and Deleeuw, 1977). Utilizing ELISA based on the monoclonal antibodies have proved its efficacy and sensitivity in detecting RV directly in fecal samples. The success of ELISA used in the current study was previously reported in similar studies on BRV in Quebec-Canada (Hussein et al.,1995 and 1996) and in Egypt (Hussein et al.,1993; and Abo Hateb et al., 2008). The assay was based on the combination of two VP6 specific monoclonal antibodies. The VP6 - ELISA used in this study has proved the presence of RV in broiler chickens diarrhea with the prevalence percentage of 15.2% demonstrating the significant occurrence of RV infection and denoted not only the importance of rotavirus infection in poultry presenting enteric problems but also lead to the isolation of chicken rotavirus field circulating strain in SPF chickens. ELISA results findings were similar to those of (Bellinzoni et al., 1987) who investigated the presence of rotavirus in a chicken flock experiencing periodic episodes of diarrhea.

The Electron microscopy continues to be very important in the detection of rotaviruses which have a distinctive morphologic appearance. Also, electron microscopy is the most rapid and confirmed diagnostic method for direct diagnosis of rotaviruses. Direct electron microscopy examination of fecal samples permits detection of rotavirus in 80% to 90% of the virus positive samples (Brandt et al., 1981 and Nakata et al., 1987). In the current study, the ELISA positive fecal samples were concentrated by ultracentrifugation then examined by direct electron microscopy and the result showed the characteristic morphological appearance of double shelled layers of rotavirus and the wheel-like appearance of the rotavirus particles Figures (1).

Virus isolation is infrequently applied to diagnose rotavirus infections as these viruses are difficult to cultivate in the laboratory (McNulty et al., 1981). The obtained results of SPF experiment revealed that the younger group demonstrated the most severe acute clinical signs specially dehydration and death faster than the middle age group while the oldest group showed the most prominent clinical signs of continuous diarrhea and was more resistant to severe dehydration and death. Indeed, the clinical signs in the experimentally infected SPF confirmed the severity of the isolated virus. These findings were similar to those reported by Yason and Schat, (1987) who indicated that older chickens more than 28 days develop humoral immunologic responses more rapidly than did younger chickens less than 21 days of age and this might to due to the short time (1 to 2 days) that virus-infected cells developed in the villi and/or to the small amount of viral antigen produced and subjected for antigen presenting cells (macrophages, dendritic cells, and M cells). Alternatively, younger birds might be immunologically immature and thereby were unable to respond to the rotavirus antigens. If development of specific IgA follows a pattern similar to that with the development of serum antibodies, repeated cycles of infection may develop before a chicken develops immunologic response that is sufficient to protect the chicken against infection by rotavirus (McFerran, 1981).

The molecular characterization of chicken RV is very important in the current study. The RT-PCR has proved its efficacy in detecting rotavirus in the fecal samples. Several other researches have used RT-PCR in detection and characterization of rotaviruses (Gouvea et al., 1993 and 1994; Isegawa et al., 1993 and Hussein et al., 1996).The obtained bands from RT-PCR were detected in the accurate size 1356 for VP6 gene indicating the detection of group A chicken rotavirus. The low number of positive samples may be due to sample storage, loss of intact virus particles, RNA instability and reaction condition of primers used. After the isolation in SPF chickens, fecal samples of SPF chickens at 4^{th} and 5^{th} days post inoculation did not give the specific band of full VP6 gene of 1348 bp when chicken set of primers (Ch1 strain specific primers) were used in the RT-PCR assay indicating that the isolated chicken rotavirus may be not belongs to the same cluster that Ch1 chicken rotavirus. However, using pigeon set of primers (Po-13 primers) revealed the specific band of 470bp not only for the fecal samples of 4^{th} and 5^{th} days post inoculation of SPF chickens but also for the original positive sample confirming the results of the characterization of a mammalian like chicken rotavirus strain from both original and SPF chickens fecal samples.

In conclusion, in current study a high frequency of rotavirus in Egyptian poultry was detected using ELISA and RT-PCR, indicating that this virus, although neglected as an important putative pathogen of poultry, may has a role in the pathogenesis of enteric disease of layers and broilers. The study highlights the implication of rotavirus in both diarrhea and the low performance of birds and should be considered as an important pathogen in a single manifestation or in association with other pathogens contributing in enteric problem onset. Indeed, the study reports for the first time the isolation of CRV from broiler flock in Egypt. Further studies on such virus are needed to be addressed.

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كشف جزيئي وعزل وتوصيف فيروس روتا الطيور من كتاكيت التسمين

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من خلال هذه الدراسة تم لأول مرة في مصر الكشف وعزل وتوصيف فيروس روتا الطيور من كتاكيت تسمين تعاني من الإسهال . تمت هذه الدراسة علي 85 عينه حقلية تم تجميعها عام 2008 من كتاكيت تسمين تعاني من الإسهال في العديد من المحافظات المصرية مثل السادس من أكتوبر – الفيوم – الجيزة – القليوبية – المنوفية – المنصورة. تم الكشف من فيروس روتا الطيور في العينات المجمعة باستخدام اختبار الاليزا المعتمد علي استخدام أجسام مناعية أحادية الفسيلة لبروتين الغلاف الداخلي (VP6) وكذلك باستخدام الميكروسكوب الالكتروني وأيضا باستخدام تفاعل البلمرة المتسلسل المسبوق بعمليه النسخ العكسي . تم عزل الفيروس من العينات الايجابية وذلك بالتمرير في الكتاكيت الخالية من المسببات المرضية . وتم تصنيف الفيروسات المعزولة جزيئيا ووجود أن الفيروسات المعزولة لا تنتمي إلي مجموعة (CH1) (الدجاج) ولكن تنتمي إلي مجموعة (PO-13) (الحمام) (شبيهة الثدييات) . ولقد أوضحت هذه الدراسة أهمية فيروسات روتا الطيور كمسببات للإسهال وتأخر النمو في كتاكيت التسمين .